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Dietary Selenium Affects Homocysteine Metabolism Differently in Fisher-344 Rats and CD-1 Mice[1–4]

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Abstract

In our previous work with rats, plasma and tissue homocysteine concentrations were decreased by selenium deprivation. The purpose of this study was to follow up and expand on that work by determining the effects of selenium status (deficient, adequate, and supranutritional) on several aspects of homocysteine metabolism involving methionine recycling and transsulfuration. A 2nd objective was to determine whether there are differences in how selenium status affects homocysteine metabolism in rats and mice. Male weanling Fischer-344 rats and male weanling CD-1 mice were fed diets containing 0, 0.2, or 2.0 μg selenium (as sodium selenite)/g for 72 d or 60 d, respectively. Plasma homocysteine and cysteine were significantly decreased by feeding rats or mice the selenium-deficient diet compared with feeding adequate or supranutritional selenium. On the other hand, plasma glutathione was increased by selenium deficiency only in rats. Also, the specific activities of liver betaine homocysteine methyltransferase and glycine *N*-methyltransferase were decreased by selenium deficiency in rats, but were unaffected by selenium status in mice. Real-time RT-PCR was used to determine the expression of the subunits of glutamate-cysteine ligase, which catalyzes the rate-limiting step in glutathione biosynthesis. The expression of Gclc, the catalytic subunit of glutamate-cysteine ligase, was upregulated by selenium deprivation in both rat and mouse liver. Gclm, the modifier subunit of glutamate-cysteine ligase, was downregulated in rats fed 2 μg Se/g compared with rats fed adequate or deficient selenium. Based on these findings, it is evident that selenium deficiency has different outcomes in mice and rats. These variables are all related to methionine/methyl metabolism. Although only one strain of rat was compared with one strain of mouse, this work suggests that differences between species may prove vital in determining which animal model is used in studies of selenium deficiency or in studies that are designed to ascertain chemopreventive mechanisms of selenium. J. Nutr. 137: 1132–1136, 2007.

Introduction

In 2000 we reported that plasma homocysteine was decreased in rats fed a selenium-deficient diet (1). However, this was not a new finding as, during the 1980s, Bunk and Combs (2) and Halpin and Baker (3) showed that free homocysteine was decreased in the plasma of selenium-deficient chicks. Additionally, studies by Hill and Burk (4) showed that selenium deficiency in rats increased plasma glutathione. They reported that this increase was the result of an upregulation of γ-glutamylcysteine synthetase [glutamate-cysteine ligase (GCL)],[7] the rate-limiting enzyme in the synthesis of glutathione (4).

In methionine metabolism, homocysteine is situated at a crossroads. Methionine (through homocysteine) can be irreversibly lost if homocysteine enters the transsulfuration pathway, the pathway that is obligatory for glutathione synthesis. However, the backbone of methionine can be reutilized if homocysteine is remethylated by 1 of 2 enzymes, methionine synthase [5-methyltetrahydrofolate-homocysteine methyltransferase (MTR), a vitamin B-12 and folate-dependent enzyme] or betaine homocysteine methyltransferase (BHMT). In 2002 we reported on a study that determined the effect of selenium in rats on various enzymes of methionine metabolism (5). We showed that plasma homocysteine

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[4] Supplemental Tables 1 and 2 are available with the online posting of this paper at jn.nutrition.org.
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[7] Abbreviations used: BHMT, betaine homocysteine methyltransferase (enzyme); Gapdh, glyceraldehyde-3-phosphate dehydrogenase; GCL, glutamate-cysteine ligase; Gclc, glutamate-cysteine ligase, catalytic subunit; Gclm, glutamate-cysteine ligase, modifier subunit; GNMT, glycine *N*-methyltransferase (enzyme); GPx, glutathione peroxidase; MTR, 5-methyltetrahydrofolate-homocysteine methyltransferase (enzyme); Mtr, 5-methyltetrahydrofolate-homocysteine methyltransferase (gene); Rn18s, 18S RNA; SAH, S-adenosylhomocysteine; SAM, *S*-adenosylmethionine.

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and cysteine were directly related to dietary selenium over a range of deficiency to adequacy (0.1 μg/g; as selenite) and that selenium deficiency resulted in an increase in plasma and liver glutathione (5). Of 5 enzymes involved with methionine metabolism that we assayed, only the activity of liver BHMT was affected by selenium; the activity of BHMT was directly proportional to dietary selenium over that range (0 to 0.1 μg Se/g diet). We found no effect of selenium on MTR activity. Thus, we concluded that the effect of selenium deprivation on homocysteine was mainly the result of upregulation of GCL and hence increased the flux of homocysteine into the transsulfuration pathway resulting in increased glutathione synthesis.

Therefore, this research was designed to follow up and expand upon our previous work to determine the effect of selenium status (deficient, adequate, and supranutritional) on several aspects of homocysteine metabolism involving remethylation to methionine and transsulfuration to cysteine and glutathione. A 2nd objective was to determine whether there are differences in how selenium status affects homocysteine metabolism in Fisher-344 rats and CD-1 mice, the animal models that we used in our selenium studies.

Materials and Methods

Animals and diets. Male, weanling Fischer-344 rats ($n = 36$) were purchased from Sasco and male weanling CD-1 mice ($n = 24$) were purchased from Charles River Laboratories. The rats and mice were weighed upon arrival and assigned to dietary group based on body weight so that there was no difference in initial weight among groups. Rats and mice were given free access to demineralized water and an amino acid–based diet in a room with controlled temperature and light. The basal amino acid–based diet (**Table 1**) was based on AIN-93 recommendations (6,7) and by analysis contained ~3 ng Se/g. An amino acid–based diet, rather than a Torula yeast-based diet, was used for 2 reasons: *1*) this diet has been used in our previous selenium studies involving folic acid deficiency (8), and *2*) to avoid mineral excesses (Fe, P, Mg, K, Zn, and Mn) that result when Torula yeast is included at concentrations (300 g/kg diet) needed to meet the protein requirement (150 g/kg diet) of growing rats (9,10).

TABLE 1 Basal diet (rats and mice)

Ingredient	*g/kg* Diet
Amino acid mix[1]	168.7
Sucrose	509.3
Soybean oil	70.0
Mineral mix[2]	20.0
Vitamin mix[3]	5.0
Alphacel fiber	50.0
Choline bitartrate	2.5
Corn starch	150.0
$CaCO_3$	7.0
KH_2PO_4	10.0
$CaHPO_4$	7.5

[1] Amino acid mix, in g/kg diet: L-arginine, 7.5; L-histidine · HCl · H_2O, 6.0; L-lysine·HCl, 16.5; L-tyrosine, 7.5; L-tryptophan, 2.5; L-phenylalanine, 7.5; L-threonine, 7.5; L-leucine, 11.0; L-isoleucine, 9.0; L-valine, 10.5; glycine, 6.0; L-proline, 4.5; L-glutamic acid, 40.0; L-alanine, 4.0; L-serine, 4.0; L-aspartic acid, 7.0; L-asparagine, 6.0; L-methionine, 8.2; L-cystine, 3.5.

[2] Mineral mix, in g/kg diet: K_2SO_4, 1.632; $CaCl_2 \cdot 2H_2O$, 1.05; Na_2CO_3, 1.5; MgO, 0.85; $Na_2SiO_3 \cdot 9H_2O$, 0.254; ferric citrate (16% Fe), 0.22; zinc carbonate (70%), 0.022; manganese carbonate (43% Mn), 0.024; cupric carbonate (55% Cu), 0.011; sucrose, 14.42; in mg/kg diet: KIO_3, 0.35; $(NH_4)_6\ Mo_7O_{24} \cdot 4H_2O$, 0.371; $CrK(SO_4)_2 \cdot 12H_2O$, 9.625; H_3BO_3, 2.853; NaF, 2.223; nickel carbonate, 2.223; SnO, 0.25; NH_4VO_3, 0.462; $NaAsO_2$, 0.4.

[3] Vitamin mix, in mg/kg diet: nicotinic acid, 30; pantothenic acid, Ca salt, 16; pyridoxine · HCl, 7; thiamine · HCl, 6; riboflavin, 6; folic acid, 2; D-biotin, 0.2; cyanocobalamin in 0.1% mannitol, 25; *dl*-α-tocopherol acetate (300 IU/g), 250; retinyl palmitate (250,000 IU/g), 16; cholecalciferol (400,000 IU/g), 2.5; phylloquinone, 1; sucrose, 4638.

Dietary treatments were addition of selenium (0, 0.2, or 2.0 μg/g), as sodium selenite, to the basal diet. Rats were on the dietary treatments for 72 d and mice for 60 d; we typically run our selenium studies anywhere from 60–72 d. This study was approved by the Animal Care Committee of the Grand Forks Human Nutrition Research Center, and the rats and mice were maintained in accordance with the guidelines for the care and use of laboratory animals.

Sample collection. Food was withheld overnight before rats and mice were anesthetized with xylazine (Rompon, Moboay) and ketamine (Ketaset, Aveco) and killed by cardiac exsanguination. Blood was collected into syringes containing EDTA such that the final concentration was ~1 g EDTA/L blood.

Selenium status. Selenium concentration in the diets was determined by hydride-generation atomic absorption spectrometry according to Finley et al. (11). Samples were prepared for analysis by predigestion in nitric acid and hydrogen peroxide, followed by high temperature ashing in the presence of $MgNO_3$ as an aid to prevent selenium volatilization. Glutathione peroxidase activity (GPx) was determined according to the coupled enzymatic method of Paglia and Valentine (12) with hydrogen peroxide (0.15 μmol/L) as the substrate.

Plasma total homocysteine, cysteine and glutathione. Total (reduced plus oxidized) homocysteine, total cysteine, and total glutathione were determined in EDTA-plasma using HPLC according to the procedure of Durand et al. (13).

Liver S-adenosylmethionine and S-adenosylhomocysteine. Portions of fresh liver were weighed and homogenized at 11,500 rpm in 0.4 mol $HClO_4$/L using a Mark II Tissumizer (Tekmar, Cincinnati, OH). Samples were centrifuged at 2000 × *g* at 4°C for 30 min. Each supernatant was filtered through a 0.45 μm filter and stored at −70°C until analysis. *S*-adenosylmethionine (SAM) and *S*-adenosylhomocysteine (SAH) were measured on a Shimadzu LC-10 HPLC equipped with a 250 × 4.6 mm Ultrasphere 5μ C18 IP column (Phenomenex) according to the procedure of Wagner et al. (14).

Liver BHMT. The activity of betaine-homocysteine methyltransferase (BHMT) was determined according to Finkelstein and Mudd (15) as modified by Xue and Snoswell (16). The substrate [methyl-^{3}H]betaine was prepared according to Xue and Snoswell (16). Liver was prepared by homogenization (1 g liver/0.004 L buffer) in 0.04 mol/L potassium phosphate buffer, pH 7.4. The homogenate was centrifuged at 18,000 × *g* for 15 min at 4°C; the supernatant was used for the assay.

Liver GNMT. The activity of liver glycine N-methyltransferase (GNMT) was determined by the method of Cook and Wagner (17). Liver was homogenized in 5 volumes of buffer as described by Cook et al. (18) and centrifuged at 100,000 × *g* for 60 min at 4°C. The supernatant fluid was diluted further, just prior to analysis (1:4 v:v supernatant fluid to 0.01 mol/L potassium phosphate buffer, pH 7.4).

Real time RT-PCR. During the animal kill and prior to freezing the liver in liquid nitrogen for enzyme analyses, a piece of liver was quickly excised and placed in RNAlater (Ambion) and then stored at −20°C until RNA extraction. Total RNA was extracted using the NucleoSpin RNA II kit (BD Biosciences). The primers used for the different genes studied were designed to include intron spanning, when possible, using the Universal Probe Library Assay Design Center (Roche Applied Science) (**Supplemental Tables 1 and 2**). Glyceraldehyde-3-phosphate dehydrogenase (Gapdh) and 18 S RNA (Rn18s) were used as reference genes for rats and mice, respectively.

Real-time RT-PCR was performed using the QuantiTect SYBR Green RT-PCR kit (Qiagen) and a SmartCycler (Cepheid) instrument. Gene expression was quantitated using the comparative C_T method (19). For the primers used, replication efficiencies of each target amplification were equal to the efficiency of the reference amplification (Gapdh or Rn18s) (data not shown). The amount of target, normalized to an endogenous reference (Gapdh or Rn18s) was expressed relative to the control group

TABLE 2 Effects in rats of dietary selenium supplementation on plasma homocysteine, cysteine, and glutathione and the activity of liver GPx[1]

Se	Homocysteine	Glutathione	Cysteine	GPx
μg/g	μmol/L			U/mg protein
0	1.53 ± 0.19[a]	7.28 ± 0.38[b]	86.7 ± 4.7[a]	25.3 ± 5.8[a]
0.2	5.57 ± 1.1[b]	5.58 ± 0.18[a]	149 ± 4.9[b]	743 ± 31[b]
2	4.12 ± 0.64[b]	5.93 ± 0.26[a]	149 ± 5.9[b]	774 ± 39[b]
Treatment effect	0.002	0.0008	0.0001	0.0001

[1] Values are means ± SEM, $n = 10$–12. Means in a column with superscripts without a common letter differ ($P < 0.05$).

(rats or mice fed 0.2 μg Se/g diet). For the amount of target in the animals fed 0 or 2.0 μg Se/g, a 1-fold of control change indicated no change, >1-fold of control change indicated upregulation, and <1-fold of control change indicated downregulation.

Statistics. Data were analyzed by 1-way ANOVA using SAS, version 9.1. For GPx, an ANOVA was done using Proc Mixed in SAS, which allowed individual group variances to be used in the analysis. Tukey's contrasts were used to differentiate among means for variables that were significantly ($P < 0.05$) affected by dietary selenium. For the real time RT-PCR data, ANOVA compared ΔC_T; however, the fold of control change ($2_T^{-\Delta\Delta C}$) was reported in the text and tables. Values are means ± SEM. Values for real-time RT-PCR are reported as means (−1 SEM, +1 SEM).

Results

The selenium status indicator, liver GPx, was markedly reduced ($P = 0.0001$) in rats (**Table 2**) and mice (**Table 3**) fed the selenium-deficient diet. GPx activity in rats or mice fed 2 μg Se/g diet did not differ from those fed an adequate amount,0.2 μg Se/g. The activity of liver GPx confirmed that the basal diet produced a marked selenium deficiency. It also showed that rats and mice fed 0.2 μg selenium/g diet had maximal enzyme activity.

Plasma homocysteine ($P \leq 0.002$) and cysteine ($P < 0.0001$) were reduced in the selenium-deprived rats and mice, compared with animals fed adequate selenium. Supplementation of 2 μg Se/g did not significantly affect plasma homocysteine or cysteine (Tables 2 and 3). Plasma glutathione was increased ($P = 0.0008$) by selenium deprivation in rats but was not affected by selenium status in mice (Table 2 and 3).

Selenium status had no effect on the concentration of liver SAM or SAH in mice (Table 3). SAM and SAH were not measured in rats because we have shown that there is little effect, if any, of selenium status on rat liver SAM and SAH (1,5,8,20).

The specific activity and expression of hepatic BHMT was decreased by selenium deprivation in rats ($P = 0.0003$ and $P = 0.0013$, respectively). The activity and mRNA content of rats fed 2 μg/g did not differ from rats fed adequate selenium (**Table 4**). In mice, however, dietary selenium had no effect on specific activity or mRNA content of BHMT (Table 4).

Compared with rats fed adequate selenium, selenium deficiency resulted in a 2-fold upregulation of the catalytic subunit of glutamate cysteine ligase (Gclc) (Table 4). Supplementation of 2 μg Se/g resulted in no change in Gclc (relative to rats fed adequate selenium). On the other hand, the modifier subunit of GCL (Gclm), was downregulated in rats fed 2 μg Se/g compared with rats fed adequate selenium (Table 4).

In mice, Gclc was upregulated (~2-fold of control) in selenium-deficient compared with selenium-adequate animals (Table 4). Supplementation of 2 μg Se/g had no effect on Gclc compared with mice fed adequate selenium. Although Gclm from both selenium-deficient and selenium-supplemented (2 μg/g) mice tended to be upregulated compared with mice fed adequate selenium, statistically there were no differences.

Expression of 5-methyltetrahydrofolate-homocysteine methyltransferase (Mtr) was not affected by dietary treatment in rats or mice (Table 4). The activity of liver GNMT was decreased by selenium deprivation in rats compared with rats fed 0.2 or 2.0 μg Se/g diet (Table 4). Dietary selenium did not affect GNMT activity in mice (Table 4).

Discussion

There are 2 major metabolic pathways involving homocysteine: remethylation and transsulfuration. In remethylation, homocysteine is converted to methionine by acquiring a methyl group from either N^5-methyltetrahydrofolate or from betaine. This conversion is catalyzed by the enzymes Mtr and BHMT, respectively. Homocysteine, through the action of cystathionine β-synthase, can also be converted to cystathionine. This 1st step in the transsulfuration pathway is irreversible. Cysteine, formed from cystathionine, can be incorporated into protein, converted to cysteinesulfinate by the enzyme cysteine dioxygenase, or be converted to γ-glutamylcysteine (the immediate precursor of glutathione) by the enzyme γ-glutamylcysteine synthetase (GCL). GCL catalyzes the rate-limiting step in glutathione synthesis.

In rats and mice there is essentially no extrahepatic BHMT (21). Because of this and because of its relative size, the liver plays a key role in methionine metabolism with remethylation and transsmethylation most likely the major pathways of methionine metabolism. Our previous work with rats also showed that the concentration of homocysteine in kidney did not differ significantly between rats fed a selenium deficient diet and those fed 0.1 μg selenium/g (5). Furthermore, total urinary homocysteine was not affected by dietary selenium (5). Thus, the present work is focused on plasma and the liver.

In the early 1980s, Bunk and Combs (2) and Halpin and Baker (3) found that free homocyst(e)ine was decreased in the plasma of selenium-deficient chicks. Furthermore, Hill and Burk showed

TABLE 3 Effects in mice of dietary selenium supplementation on plasma homocysteine, cysteine and glutathione, the activity of liver glutathione peroxidase, and the hepatic concentrations of SAH and SAH[1]

Se	Homocysteine	Glutathione	Cysteine	GPx	SAM	SAH
μg/g	μmol/L			U/mg protein	nmol/g	
0	1.26 ± 0.10[a]	8.63 ± 0.77	80.4 ± 4.6[a]	18.8 ± 3.2[a]	83.5 ± 9.0	17.0 ± 1.2
0.2	3.44 ± 0.68[b]	9.41 ± 0.78	143 ± 9.3[b]	1101 ± 30[b]	83.1 ± 3.8	18.3 ± 0.78
2	3.69 ± 0.30[b]	9.24 ± 0.86	144 ± 8.0[b]	1057 ± 36[b]	90.1 ± 2.7	18.2 ± 0.98
Treatment effect	0.0012	0.77	0.0001	0.0001	0.70	0.63

[1] Values are means ± SEM, $n = 8$/group. Means in a column with superscripts without a common letter differ, $P < 0.05$.

TABLE 4 The effect, in rat and mouse liver, of dietary selenium supplementation on the activity and expression of BHMT, Gclc, Gclm, Mtr, and the activity of GNMT[1]

Se treatment	BHMT	Bhmt	Gclc	Gclm	Mtr	GNMT
μg/g	*nmol · min*$^{-1}$ *· mg protein*$^{-1}$	*fold of control*[2]				*nmol · min*$^{-1}$*·mg protein*$^{-1}$
Rat						
0	1.79 ± 0.07[a]	0.56[a] (0.47, 0.66)	1.92[a] (1.73, 2.12)	1.29[a] (1.16, 1.44)	0.80 (0.64, 1.00)	0.723 ± 0.052[a]
0.2	2.29 ± 0.09[b]	1.00[b] (0.91, 1.10)	1.00[b] (0.93, 1.08)	1.00[a] (0.92, 1.09)	1.00 (0.84, 1.20)	0.910 ± 0.036[b]
2	2.29 ± 0.10[b]	0.99[b] (0.87, 1.12)	1.05[b] (0.95, 1.15)	0.74[b] (0.67, 0.82)	1.10 (0.92, 1.32)	0.914 ± 0.063[b]
Treatment effect	0.0003	0.0013	0.0001	0.0001	0.32	0.023
Mouse						
0	3.75 ± 0.33	1.07 (0.73, 1.55)	1.75[a] (1.41, 2.19)	1.26 (1.01, 1.57)	1.01 (0.66, 1.54)	2.61 ± 0.13
0.2	4.08 ± 0.53	1.00 (0.68, 1.47)	1.00[b] (0.88, 1.34)	1.00 (0.85, 1.17)	1.00 (0.76, 1.31)	2.73 ± 0.13
2	3.54 ± 0.44	1.00 (0.63, 1.58)	1.22[ab] (1.04, 1.42)	1.32 (1.13, 1.54)	1.33 (0.96, 1.85)	2.60 ± 0.11
Treatment effect	0.69	0.98	0.048	0.34	0.70	0.68

[1] Values are means ± SEM; $n = 10$–12 for rat data; $n = 6$–8 for mouse data; except BHMT, where $n = 5$. For each species, means in a column with superscripts without a common letter differ, $P < 0.05$.
[2] (−1 SEM, +1 SEM); control group = 0.2 μg Se/g.

that, in rats, liver glutathione production is increased and plasma glutathione is markedly elevated by selenium deficiency (4,22–24). They also reported that the activity of GCL in the selenium-deficient rat liver was twice that found in controls (4). GCL, in its most catalytically active form, is composed of a catalytic subunit (Gclc) and a modifier subunit (Gclm) (25). Chen et al. (25) found that Gclm was limiting (relative to Gclc) in mouse liver, lung, and brain, but not kidney, and that, as the molar ratio of Gclm:Gclc increased, the velocity of γ-glutamylcysteine formation increased. In our study, hepatic Gclc expression was upregulated by selenium deficiency compared with animals fed adequate selenium, in both rats and mice. Gclm expression was increased by selenium deficiency in rats. However, in mice, because of the large variability, Gclm only tended to be increased by selenium deprivation. Notably, both rats and mice had decreased concentrations of plasma homocysteine when fed a selenium-deficient diet; however, plasma glutathione was only increased by selenium deficiency in rats, but not mice. Thus, consideration must be given to more than just upregulation of GCL and the "pulling" of homocysteine into the transsulfuration pathway. We found that hepatic Mtr expression was not affected by selenium deficiency in rats or mice. Hence, the decrease in homocysteine seen in rats and mice is not the result of differences in Mtr expression. However, considering that BHMT (activity and expression) is decreased in rats (but not mice) fed a selenium-deficient diet, it is possible that more homocysteine is methylated in mice than in rats. Conversely, less homocysteine would be methylated by BHMT in rats. Because inhibition of BHMT has been shown to increase plasma homocysteine (26), one would expect that selenium-deficient rats would have higher levels of plasma homocysteine than selenium-deficient mice. Because the selenium-deficient rats have similar plasma homocysteine concentrations as selenium-deficient mice, another factor must be taken into consideration. In both animals, cysteine was significantly decreased by selenium deprivation. Cysteine is generally the rate-limiting substrate for GCL (27) and hence glutathione biosynthesis. However, glutathione is only increased in the selenium-deficient rats, not mice. Insofar as glutathione serves as a reservoir of available cysteine (27), this would suggest that more glutathionine is hydrolyzed to maintain a basal level of cysteine in selenium-deficient mice.

Finally, we confirmed that GNMT is decreased by selenium deprivation (Table 4). We have shown in rats that this change in GNMT does not correlate with changes in hepatic SAM or SAH. Selenium, however, had no effect on GNMT or SAM and SAH in mice. GNMT is an enzyme important to the regulation of tissue concentrations of *S*-adenosylmethionine and *S*-adenosylhomocysteine (17,28–31). In rats, GNMT is a major hepatic enzyme that makes up between 0.5 and 1% of the soluble protein (31). Presently, we are unable to determine the importance of the changes in GNMT caused by selenium deprivation in rats. However, these are interesting findings in light of the evidence that GNMT expression and activity are decreased in tumor tissue and that GNMT has been classified as a tumor susceptibility gene (32–35).

In this study, we compared only male CD-1 mice to male Fisher-344 rats fed identical diets. However, based on plasma glutathione and cysteine and liver BHMT and GNMT (i.e., parameters related to methionine/methyl metabolism), it is evident that selenium deficiency can result in different outcomes based on the animal model selected. Thus, species differences may prove vital in determining the animal model used in studies of selenium deficiency or in studies designed to ascertain the chemopreventive mechanisms of selenium.

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